Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

Vinood B. Patel
Victor R. Preedy *Editors*

Biomarkers in Cardiovascular Disease

Springer Reference

Biomarkers in Disease: Methods, Discoveries and Applications

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In the past decade there has been a sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. Furthermore, the discovery of many new markers have not been put into clinical practice, partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. In some cases the work needs further scientific scrutiny. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of *Biomarkers in Disease*: *Methods, Discoveries and Applications*. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

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Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Cardiovascular Disease

With 213 Figures and 138 Tables

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Volume Preface

In the present volume, ***Biomarkers in Cardiovascular Disease***, we have sections on

- **General Aspects**
- **Circulating and Body Fluid Biomarkers**
- **Specific Diseases and Conditions**
- **Molecular, Cellular, and Histological Variables**
- **Functional and Structural Variables**

While the Editors recognize the difficulties in assigning particular chapters to particular sections, the book has enormously wide coverage and includes the following areas, analytes, and conditions: testing pharmacological profiles, multiple biomarkers, use in functional foods, the extracellular matrix and collagen, PCSK9, vasoactive peptide urotensin II, fetuin-A, cholinesterase, triglycerides, high density lipoprotein-c, heart-type fatty acid binding protein (H-FABP), uncarboxylated matrix Gla protein, microRNAs, troponin, vascular endothelial growth factor-1, macrophage metalloprotease (MMP)-12, homocysteine, neutrophil gelatinase associated lipocalin (NGAL), testosterone and dihydrotestosterone, leukotrienes, 8-isoprostane, irisin, adiponectin, lipids and lipoproteins, gamma glutamyltransferase (GGT), plasma factor VIII levels, RhoA/Rho-associated kinase, polymorphisms in the vitamin D pathway, nitric oxide regulating proteins, genomics and proteomics, stem cells, virtual histology (VH), coronary plaque composition, pulse pressure and pulse pressure amplification, ventricular activation time, neutrophils, computed tomography, histology, blood flow velocity, myocardial blood, cerebral blood flow, functional transcranial Doppler ultrasound, epicardial fat thickness, electrocardiographic markers, J wave and fragmented QRS formation, intravascular ultrasound, and magnetic resonance, atrial fibrillation, chronic heart failure, abdominal aortic aneurysm, arrhythmias, resynchronization therapy, venous thromboembolism, carotid artery stenting, coronary artery disease, sudden cardiac death, diabetes, cirrhosis, and portal hypertension.

There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts and specialist. This book is specifically designed for clinical biochemists, cardiologists, cardiovascular health scientists, epidemiologists, and doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

April 2015 Vinood B. Patel
London Victor R. Preedy

Series Preface

In the past decade, there has been a sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics, leading to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology, immunoassays, or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Potential Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to

other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Series is expected to prove useful for clinicians, scientists, epidemiologists, doctors, and nurses, and also academicians and students at an advanced level.

April 2015 Victor R. Preedy
London

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About the Editors

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Dr. Vinood B. Patel, **B.Sc., Ph.D., FRSC** is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in the pathophysiology of liver disease. Other areas of interest are identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimer's disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health prevention, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy B.Sc., Ph.D., D.Sc., FRSB, FRSH, FRIPHH, FRSPH, FRCPath, FRSC is a senior member of King's College London (Professor of Nutritional Biochemistry) and King's College Hospital (Professor of Clinical Biochemistry; Honorary). He is attached to both the Diabetes and Nutritional Sciences Division and the Department of Nutrition and Dietetics. He is also founding and

current Director of the Genomics Centre and a member of the School of Medicine. Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree, for his contribution to the science of protein metabolism in health and disease. Professor Preedy was elected as a Fellow of the Institute of Biology (Society of Biology) in 1995 and to the Royal College of Pathologists in 2000. He was then elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health and Hygiene (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In 2015, the Society of Biology received its Royal Charter, so Professor Preedy became an FRSB. In his career, Professor Preedy worked at the National Heart Hospital (part of Imperial College London) and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on biomedical sciences and has a long-standing interest in analytical methods and biomarkers, especially their applications to the study of health and disease. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 500 articles, which includes peer-reviewed manuscripts based on original research, reviews, abstracts, and numerous books and volumes.

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Part I

General Aspects

Testing Pharmacological Profiles with Biomarkers Relevant to Cardiovascular Profiles

1

Giuseppe Derosa and Pamela Maffioli

Contents

Abstract

Cardiovascular diseases are the leading cause of morbidity and mortality in the United States and in Europe. Early identification of biomarkers linked to cardiovascular disease can be effective in identifying high-risk patients to early treat them in order to reduce cardiovascular diseases. The aim of this chapter is to

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examine biomarkers relevant to cardiovascular diseases in order to identify targets where pharmacological treatment can act. In this chapter, we will examine the main markers of insulin resistance, inflammation, endothelial damage, and metabolism linked to cardiovascular diseases.

Keywords

Adhesion molecules • Biomarkers • Cardiovascular diseases • Inflammation • Insulin resistance

Abbreviations

ADN	Adiponectin
Apo A-I	Apolipoprotein A-I
Apo B	Apolipoprotein B
C-IMT	Carotid intima-media thickness
DBP	Diastolic blood pressure
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
Hs-CRP	High-sensitivity C-reactive protein
IL-6	Interleukin-6
Lp(a)	Lipoprotein(a)
MMP-2	Metalloproteinase-2
MMP-9	Metalloproteinase-9
MPO	Myeloperoxidase
PAI-1	Plasminogen activator inhibitor-1
PON-1	Paraoxonase-1
PPG	Postprandial glucose
RBP-4	Retinol binding protein-4
SBP	Systolic blood pressure
sE-selectin	Soluble E-selectin
sICAM	Serum intracellular adhesion molecule-1
sVCAM	Soluble vascular cell adhesion molecule-1
TNF-α	Tumor necrosis factor-α

Key Facts of Cardiovascular Diseases

- Cardiovascular diseases include a group of conditions that involve ischemic heart disease, stroke, and peripheral artery disease.
- The underlying mechanism of cardiovascular diseases involves atherosclerosis.
- There are factors accelerating atherosclerosis; they include smoke, physical inactivity, diabetes, weight gain, elevated blood pressure, and inadequate lipid profile.
- In order to prevent atherosclerosis, early identification and correction of these risk factors is very important.

- When identified, risk factors should be corrected; in particular, we can improve lipid profile using hypocholesterolaemic agents, reduce glycaemia with antiglycaemic agents, and induce weight decrease with a well-balanced diet.

Definitions

Adipocytokines A family of bioactive products secreted by adipose tissue; adipocytokines include inflammatory mediators, angiogenic proteins, and metabolic regulators.

Atherosclerosis Atherosclerosis is a process in which an artery wall thickens as a result of chronic inflammatory response to cholesterol deposit.

Biomarker The term refers to a measurable indicator of some biological state or condition that can be used for diagnosis or follow-up a particular disease.

Endothelial dysfunction It is a systemic pathological state of the endothelium in response to cardiovascular risk factors and precedes the development of atherosclerosis.

HOMA index Is a method used to quantify insulin resistance and β-cell function; it is calculated with a formula that considers glycaemia and insulinemia.

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in the United States and in Europe, and for this reason primary and secondary prevention of cardiovascular diseases are public health priorities (Pearson et al. 2002). In this regard, biomarkers are one tool to better identify high-risk patients, in order to promptly and accurately diagnose diseases and to effectively prognosticate and treat patients.

Biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). Biomarkers may also serve as surrogate end points. A surrogate end point is one that can be used as an outcome in clinical trials to evaluate safety and effectiveness of therapies instead of measurement of the true outcome of interest. The underlying principle is that alterations in the surrogate end point track closely with changes in the outcome of interest (Colburn 2000; De Gruttola et al. 2001). Surrogate end points have the advantage that they may be gathered in a shorter time frame and with less expense than end points such as morbidity and mortality, which require large clinical trials for evaluation.

In this regard, the aim of this chapter is to examine biomarkers relevant to cardiovascular disease in order to identify targets where pharmacological treatment can act in order to give practical considerations useful for the clinical practice.

Description of the Main Biomarkers Relevant to Cardiovascular Profiles

Markers of Insulin Resistance

Insulin resistance is a hallmark of obesity, diabetes, and cardiovascular diseases and leads to many of the abnormalities associated with metabolic syndrome. Insulin resistance is established by genetic and environmental factors. Insulin resistance leads to impaired glucose tolerance and plays an important pathophysiological role in the development of diabetes (DeFronzo et al. 1992). Patients with insulin resistance are likely to have impaired fasting plasma glucose levels, which, in turn, enhance the prevalence of more atherogenic, small dense low-density lipoprotein (LDL) particles. Central obesity and insulin resistance form the basis of the pathophysiology of dyslipidaemia, lack of glucose tolerance, and the existence of chronic subclinical inflammation and hypertension in metabolic syndrome.

The markers of insulin resistance are listed in Table 1 and include:

- *Bioumoral markers*: glycaemia, fasting plasma insulin, HOMA index, small and dense LDL
- *Adipocytokines*: adiponectin, resistin, visfatin, vaspin, retinol binding protein-4 (RBP-4)
- *Fat*: subcutaneous fat, visceral fat, epicardial fat

Bioumoral Markers

The quantification of insulin resistance can be performed by evaluating the peripheral insulin sensitivity in vivo with methods such as the pancreatic suppression test (Greenfield et al. 1981), the hyperinsulinemic-euglycaemic clamp technique (Greenfield et al. 1981), and the minimal model approximation of the metabolism of glucose (MMAMG) (Bergman et al. 1985). They are complicated, time-consuming, and expensive methods suitable only for studies with a small number of subjects. For epidemiologic and clinical studies, simpler, indirect methods have been advocated for quantification of insulin resistance, based on measuring plasma insulin levels during fasting or after glucose stimulus and on the insulin-glucose ratio calculated with different mathematical formulas. Such methods include measurement of fasting plasma insulin levels and 2-h post-75-g oral glucose load, the homeostasis model assessment (HOMA) (Matthews et al. 1985), and the mathematical calculations known as the quantitative insulin sensitivity check index (QUICKI) (Katz et al. 2000) and McAuley et al. (2001) indexes. Among these indexes, the HOMA index has been validated with the hyperinsulinemic-euglycaemic clamp technique (Bonora et al. 2000), and, therefore, it is considered a valid method to assess

Table 1 Markers of insulin resistance

Bioumoral markers	Adipocytokines	Fat
Glycaemia	Adiponectin	Subcutaneous fat
Fasting plasma insulin	Resistin	Visceral fat
HOMA index	Visfatin	Epicardial fat
Small and dense LDL	Vaspin	
	Retinol binding protein-4	

HOMA Index = [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/405

or

HOMA Index = [fasting plasma glucose (mmol/l) x fasting plasma insulin (mU/l)]/22.5

QUICKI index = 1/[log(fasting plasma insulin (μU/mL) + log(fasting plasma glucose (mg/dL)]

McAuley index = exp[2.63-0.28 ln(fasting plasma insulin (mU/l)) - 0.31 ln(triglycerides (mmol/l))]

Fig. 1 Formulas to calculate indexes of insulin resistance

peripheral insulin sensitivity in epidemiologic studies (Ascaso et al. 2003). To see how these indexes can be calculated, see Fig. 1.

Regarding small and dense LDL, an increased concentration of LDL cholesterol is widely recognized as a risk factor for coronary heart disease (Castelli et al. 1986). There is considerable heterogeneity in the size and density of LDL particles (Shen et al. 1981). Austin et al. (1988) found that most individuals can be assigned to one of two LDL subclass patterns (A or B). Small, dense LDL particles (pattern B) are thought to be more atherogenic than larger LDL particles. Reaven et al. (1993) showed that subjects with a preponderance of small, dense LDL particles (pattern B) were more insulin resistant and had higher triglyceride concentrations than subjects with larger LDL particles (pattern A). This was confirmed also by Mykkänen et al. that concluded that a preponderance of small, dense LDL particles is associated with insulin resistance and that serum triglycerides concentration modifies this relationship (Mykkänen et al. 1997), implying that triglycerides levels are a contributing factor to insulin resistance.

Adipocytokines

It is now clear that adipose tissue is a complex and highly active metabolic and endocrine organ. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells. Although adipocytes

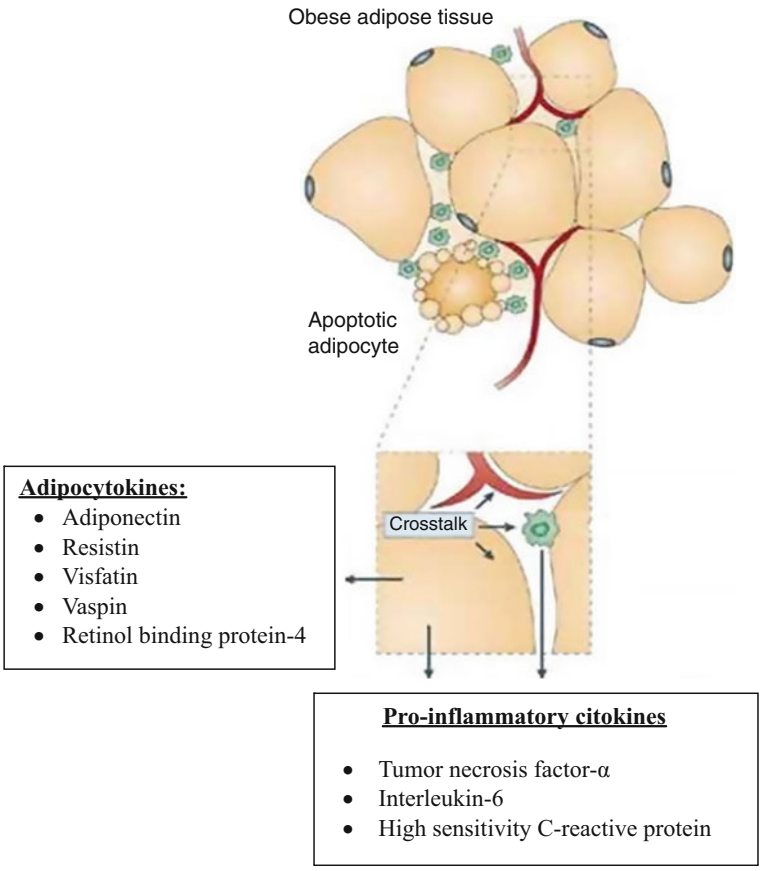

Fig. 2 Mechanism of insulin resistance. (Adapted from Tilg et al. 2006).

express and secrete several endocrine hormones such as leptin and adiponectin, many secreted proteins are derived from the non-adipocyte fraction of adipose tissue. Regardless, these components function as an integrated unit, making adipose tissue a true endocrine organ (Fig. 2).

Adiponectin is a protein exclusively synthesized by adipocytes; it is decreased in obesity and inversely related to glucose and insulin. Ablation of the adiponectin gene in mice resulted in insulin resistance, glucose intolerance, dyslipidaemia, and increased susceptibility to vascular injury and atherosclerosis (Berg et al. 2002). Adiponectin reverses these abnormalities by stimulating oxidation of fatty acids, suppressing gluconeogenesis and inhibiting monocyte adhesion, macrophage transformation, and proliferation and migration of smooth muscle cells in blood vessels (Berg et al. 2002).

Human resistin, instead, is secreted by mononuclear cells and activated macrophages (Steppan et al. 2001) and was named for its ability to induce insulin resistance in rodents. Resistin has been linked to obesity and diabetes, and it has

been reported to be elevated in adipose tissue and serum in obesity and insulin resistance (Azuma et al. 2004). Moreover, resistin appears to confer an increased risk of inflammation and atherosclerosis (Silswal et al. 2005).

Visfatin was discovered as a secretory protein highly enriched in rodent and human visceral adipocytes (Fukuhara et al. 2005). The expression and secretion of visfatin is increased during the development of obesity; however, in contrast with inflammatory cytokines, the rise in visfatin does not decrease insulin sensitivity. Instead, visfatin exerts insulin-mimetic effects in cultured adipocytes, hepatocytes, and myotubes and lowers plasma glucose in mice. Visfatin binds to the insulin receptor with similar affinity but at a site distinct from insulin (Fukuhara et al. 2005). In contrast with insulin, visfatin levels do not change with feeding and fasting (Fukuhara et al. 2005). The discovery of visfatin may lead to novel therapies for diabetes; however, it remains to be determined if visfatin acts in concert with insulin to regulate metabolism and whether such interaction occurs via endocrine or paracrine mechanisms. Vaspin (visceral adipose tissue-derived serpin) is a member of the serine protease inhibitor family isolated by from the visceral white adipose tissue of OLETF (Otsuka Long-Evans Tokushima fatty) rats, a model of abdominal obesity, insulin resistance, and diabetes (Hida et al. 2005). Administration of vaspin to obese mice improves glucose tolerance and insulin sensitivity and reverses the expression of half of the number of genes induced in white adipose tissue by diet-induced obesity (Hida et al. 2005).

Finally, RBP-4, the sole retinol transporter in blood, is secreted from adipocytes and liver. Serum RBP-4 levels correlate highly with insulin resistance, other metabolic syndrome factors, and cardiovascular disease. Elevated RBP-4 levels cause insulin resistance in mice and humans (Yang et al. 2005), but the molecular mechanisms are unknown. Probably RBP-4 induces expression of proinflammatory cytokines in mouse and human macrophages and, thereby, indirectly inhibits insulin signaling in cocultured adipocytes. Studies in mice suggest that adipose tissue serves as a glucose sensor and regulates systemic glucose metabolism through release of RBP-4 in response to decreased intracellular glucose concentrations (Yang et al. 2005).

Fat

Fat accumulates mainly in subcutaneous depots, but sizeable amounts of adipose tissue are also deposited in the abdomen (between and within organs), in the thorax (as epicardial, mediastinal and/or intramyocardial fat), in the pancreas, and in skeletal muscle. Although the impact of central versus peripheral body fat on both metabolic and cardiovascular dysfunction has been firmly established, the importance of the site of abdominal fat accumulation in relation to insulin sensitivity is still a matter of some debate. Some studies have suggested that the intra-abdominal fat depot is the major determinant of insulin resistance (Canepa et al. 2013; Park et al. 1991), and of other features of the metabolic syndrome (Després, et al. 1989), whereas others have suggested that the subcutaneous fat compartment is the most critical determinant of insulin sensitivity (Mazurek et al. 2003). Several methods of assessing the amount of visceral fat accumulation have been investigated; they can be helpful to estimate not only visceral obesity but also the risk of cardiovascular and metabolic diseases.

The simplest way to assess visceral fat is to use an anthropometric index such as body mass index, waist circumference, waist hip ratio, abdominal sagittal diameter, or neck circumference. These values provide a fast, easy, and noninvasive method of assessing regional adiposity, particularly in epidemiologic studies. However, it is possible that substantial variations in the visceral fat content may be observed among persons with a similar waist circumference or waist hip ratio value, because these indexes are not the direct methods of quantifying the amount of fat or of discriminating between visceral and subcutaneous fat. Accordingly, an alternative and reliable method can be assessment of visceral fat thickness by ultrasonography. Using this technique, subcutaneous and visceral fat diameters can be measured using a 3.0 MHz curved array transducer. Visceral adipose tissue diameter can be measured from the internal surface of the rectus abdominis muscle to the near wall of the aorta. Subcutaneous adipose tissue diameter can be measured at the same position as the distance between the external surface of the muscle and the skin. The thickness of the muscle and skin need to be excluded.

Also cardiac fat is now recognized as a new cardiometabolic risk marker, as it is associated with increased insulin resistance, cardiovascular risk factors, visceral fat, and, in general, with the metabolic syndrome. In the supradiaphragmatic region, fat is deposited in the intrathoracic space (extra-pericardial adipose tissue or mediastinal fat), around the myocardium (epicardial adipose tissue) and as intramyocardial fat. The few data available in literature suggest that epicardial fat can be an important determinant of diastolic dysfunction (Abate et al. 1995). Because epicardial adipose tissue secretes proinflammatory, proatherogenic, and prothrombotic adipokines (Dutour et al. 2010) and there is no physical barrier separating it from the adjacent myocardium and coronary arteries, epicardial adipose tissue can have a local metabolic role by a paracrine effect (Sacks and Fain 2007).

Inflammatory Markers

Several older prospective epidemiological studies have documented an association between inflammatory markers such as white blood cells count and fibrinogen (Yarnell et al. 1991) and cardiovascular disease. Since these publications, a large number of peer-reviewed scientific reports have been published relating inflammatory markers to cardiovascular disease. In the latest years, several commercial assays to assess inflammatory markers have become available. As a consequence of the expanding research base and availability of assays, the number of inflammatory marker tests ordered by clinicians for cardiovascular disease risk prediction has grown rapidly.

The inflammatory markers are listed in Table 2, and include:

- *Bioumoral markers*: white blood cells, platelets, fibrinogen.
- *Cytokines*: tumor necrosis factor-α (TNF-α), high-sensitivity C-reactive protein (Hs-CRP), myeloperoxidase (MPO), interleukin-6 (IL-6), paraoxonase-1 (PON-1).

Table 2 Inflammatory markers

Bioumoral markers	Cytokines
White blood cells	Tumor necrosis factor-α
Platelets	High-sensitivity C-reactive protein
Fibrinogen	Myeloperoxidase
	Interleukin-6
	Paraoxonase-1

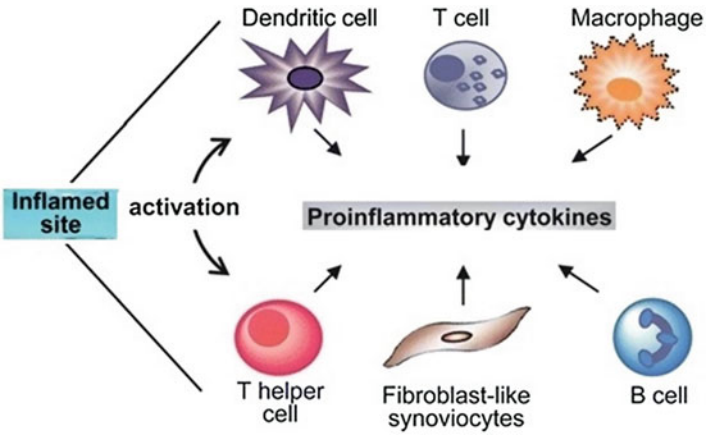

Fig. 3 Mechanism of inflammation. (Adapted from Pan et al. 2010).

Bioumoral Markers

An elevated total white blood cell count is a risk factor for atherosclerotic vascular disease. White blood cell-derived macrophages and other phagocytes are believed to contribute to vascular injury and atherosclerotic progression (Fuster and Lewis 1994) (Fig. 3). Several prospective studies have shown a positive and independent association between white blood cell count and coronary heart disease incidence or mortality (Folsom et al. 1997; de Labry et al. 1990). The presence of leukocytes within atherosclerotic arteries was reported in the early 1980s. Initially, investigators thought that only macrophages were predominantly present within atherosclerotic vessels; however, several studies reported the presence of most known leukocytes in both mouse and human aortas. A key initiating process of atherosclerosis is the intimal retention of apolipoprotein B (Apo B) containing lipoproteins in regions of disturbed blood flow and low shear stress. In response to intimal lipid accumulation, disturbed blood flow, low shear stress, and other stimuli, endothelial cells permit monocytes, major precursors of macrophages, passage across the endothelium. Newly-infiltrated monocyte-derived macrophages recognize and ingest lipids that have accrued in the intima as a consequence of hypercholesterolemia. Macrophages are specialized phagocytes that rely on different strategies to sense, internalize, and process the diverse lipid moieties they encounter. Lipoprotein recognition and consequent ingestion morphs macrophages into lipid-rich macrophages, known as

foam cells, many of which eventually die and contribute to a large lipid core, a characteristic of lesions most vulnerable to rupture. In experimental atherosclerosis, neutrophils, whose numbers rise in the blood, help monocyte adhesion or transmigration by releasing alarmins and other preformed granular proteins. Neutrophils also contain large quantities of myeloperoxidase, NADPH oxidase, and lipoxygenases, which contribute to oxidative stress, a major determinant of endothelial cell dysfunction, lesion growth, and instability of plaque. Also mast cells promote atherosclerosis by releasing the contents of their protease-cytokine-autacoid-rich granules.

Platelets also play an important role. In normal situations, platelets can detect a disruption in the lining of a blood vessel and react to build a wall to stop bleeding. In cardiovascular disease, abnormal clotting occurs resulting in heart attacks or stroke. During atherosclerosis, blood vessels injured by smoking, cholesterol, or high blood pressure develop cholesterol-rich plaques that line the blood vessel; these plaques can rupture and cause the platelets to form a clot (Gregg and Goldschmidt-Clermont 2003). During plaque rupture, they form the thrombus that causes ischemia of downstream tissue in heart attacks, strokes, and peripheral vascular disease.

Fibrinogen is a protein produced by the liver; it usually helps stop bleeding by helping blood clots to form. However, fibrinogen has been identified as an independent risk factor for cardiovascular disease and associated with traditional cardiovascular risk factors (Lee et al. 1993), suggesting that elevation of fibrinogen may be a pathway by which these risk factors exert their effect. There are several mechanisms by which fibrinogen may increase cardiovascular risk. First, it binds specifically to activated platelets via glycoprotein IIb/IIIa, contributing to platelet aggregation. Second, increased fibrinogen levels promote fibrin formation. Third, fibrinogen is a major contributor to plasma viscosity. Finally, it is an acute-phase reactant that is increased in inflammatory states (Stec et al. 2000).

Cytokines

Among many inflammatory markers, TNF-α emerged as a key cytokine that influences intermediary metabolism. TNF-α is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as monocytes, T-cells, smooth muscle cells, adipocytes, and fibroblasts. Tumor necrosis factor-α has important proinflammatory properties, which play crucial roles in the innate and adaptive immunity, cell proliferation, and apoptotic processes (Popa et al. 2007). Tumor necrosis factor-α also plays a crucial role in the development of atherosclerotic lesions. In addition, TNF-α is able to induce proatherogenic lipoprotein changes. Finally, TNF-α, by decreasing insulin sensitivity, contributes to the development of glucose metabolism disturbances (Popa et al. 2007).

As already said above, inflammation plays a major role in the initiation, progression, and destabilization of atheromas. Laboratory and experimental evidence indicate that atherosclerosis, in addition to being a disease of lipid accumulation, also represents a chronic inflammatory process. Thus, researchers have hypothesized that

inflammatory markers such as Hs-CRP may provide an adjunctive method for global assessment of cardiovascular risk. High-sensitivity C-reactive protein is an acute-phase reactant, produced predominantly in hepatocytes as a pentamer of identical subunits in response to several cytokines; it is a circulating acute-phase reactant that reflects active systemic inflammation. An association of Hs-CRP with risk for cardiovascular disease has been described in many studies, the Multiple Risk Factor Intervention Trial was the first of many primary prevention, prospective epidemiological studies to show a strong relationship between levels of Hs-CRP and mortality from coronary heart disease in high-risk middle-aged men (Kuller et al. 1996).

High-sensitivity C-reactive protein rises acutely after tissue injury, including myocardial infarction. Intense cytokine production and inflammatory cell infiltration occurs in the area of ischemia and necrosis. This increase in Hs-CRP, in part, correlates with infarct size and with a higher risk of cardiac rupture.

Myeloperoxidase is a leukocyte-derived enzyme that catalyzes the formation of a number of reactive oxidant species. In addition to being an integral component of the innate immune response, evidence has emerged that MPO-derived oxidants contribute to tissue damage during inflammation and atherosclerosis. Myeloperoxidase has been linked to activation of protease cascades and both proapoptotic and prothrombotic pathways that are believed to be involved in plaque fissuring development of superficial erosions and intracoronary thrombus generation during sudden cardiac death (Fu et al. 2001; Baldus et al. 2004).

Increased levels of IL-6 have been also associated with high risk of all-cause mortality in older people. Interleukin-6 plays an important role in mediating inflammation and is a central stimulus for the acute-phase response (Papanicolaou et al. 1998). In particular, IL-6 induces the hepatic synthesis of C-reactive protein (CRP), described above as a known proinflammatory marker of atherothrombotic vascular disease.

Serum PON-1 is an HDL-associated lipo-lactonase, which is synthesized and secreted by the liver. Several lines of clinical and experimental evidence strongly support a potential role for PON-1 in protection against atherosclerosis. Paraoxonase-1 has anti-atherogenic properties, which are associated with the enzyme's capability to protect LDL, as well as HDL from oxidation, to decrease macrophage oxidative status, to stimulate cholesterol efflux from macrophages, and to decrease oxidative status in atherosclerotic lesions. Furthermore, PON-1 was suggested to contribute to the anti-inflammatory activity of HDL by destroying biologically active lipids in mildly oxidized LDL, resulting in decreased inflammatory responses in the artery wall cells. Moreover, PON-1 was shown to decrease monocyte chemotaxis and adhesion to endothelial cells, to inhibit monocyte-to-macrophage differentiation, and the absence of PON-1 was shown to be associated with overexpression of adhesion molecules (Aharoni et al. 2013). Experiments with transgenic PON-1 knockout mice confirm the potential for PON-1 to protect against atherogenesis. Oxidation of serum low density lipoproteins (LDL) is an important early step in the development of atherosclerosis. The oxidized products are scavenged by macrophages which eventually transform into foam cells, filled with cholesterol esters. They eventually become fatty streaks in the endothelium and

Table 3 Endothelial damage markers

Bioumoral markers	Cellular adhesion molecules	Matrix metalloproteinases	Clinical markers
Postprandial dyslipidaemia	Soluble intracellular adhesion molecule-1	Metalloproteinases-2	Systolic and diastolic blood pressure
Microalbuminuria	Soluble vascular cell adhesion molecule-1	Metalloproteinases-9	Carotid intima-media thickness
Nitrites/nitrates	sE-selectin		
Plasminogen activator inhibitor-1			

finally are seen as atheromatous plaques. Mackness was the first to suggest that serum PON-1 may be able to protect against the initial stage of this process, the oxidation of the LDL phospholipids (La Du et al. 1999).

Endothelial Damage Markers

Endothelial dysfunction is a well established response to cardiovascular risk factors and precedes the development of atherosclerosis. Endothelial dysfunction is involved in lesion formation by the promotion of both the early and late mechanisms of atherosclerosis including upregulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced low-density lipoprotein oxidation, platelet activation, cytokine elaboration, and vascular smooth muscle cell proliferation and migration. Endothelial dysfunction is a term that covers diminished production/availability of nitric oxide and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors (Hadi et al. 2005).

Endothelial damage markers are listed in Table 3 and include:

- *Bioumoral markers*: postprandial dyslipidaemia, microalbuminuria, nitrites/nitrates, plasminogen activator inhibitor-1 (PAI-1)
- *Cellular adhesion molecules*: soluble intracellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble E-selectin (sE-selectin)
- *Matrix metalloproteinases*: metalloproteinases-2 and -9 (MMP-2 and -9)
- *Clinical markers*: systolic and diastolic blood pressure, carotid intima-media thickness (C-IMT)

Bioumoral Markers

In people with diabetes or insulin resistance, the incidence of dyslipidaemia is high, and lipoprotein abnormalities play a key role in the development of atherosclerotic vascular complications. Increased postprandial lipidaemia is a characteristic aspect of diabetic dyslipidaemia; it has a high prevalence among people with diabetes even

when they have normal fasting triglycerides levels. Postprandial hyperlipidaemia exerts a relevant effect on the development of diabetic macrovascular complications since the excessive increment of triglycerides and their persistence in the circulation is accompanied by deviations towards a more atherogenic metabolism, with accumulation of remnants and small, dense LDL. These are more easily oxidized than large buoyant LDL (Rebolledo and Actis Dato 2005). The most adequate way to experimentally reproduce the postprandial lipemia condition appears to be the administration of a standardized oral fat load (OFL) to fasting patient. Clinical studies confirmed that OFL gave a reduction of nitrites/nitrates and ADN and increase MMP-2 and MMP-9 (Derosa et al. 2010a). At the same time, OFL induced a complex and massive systemic inflammatory response that included IL-6, TNF-α, Hs-CRP, and cell adhesion molecules, even before triglycerides significantly rose (Derosa et al. 2009).

Microalbuminuria is a strong and independent indicator of increased cardiovascular risk among individuals with and without diabetes. Therefore, microalbuminuria can be used for stratification of risk for cardiovascular disease. Once microalbuminuria is present, cardiovascular risk factor reduction should be more "aggressive" (Stehouwer and Smulders 2006). Dinneen and Gerstein (Dinneen and Gerstein 1997), in a systematic review, showed microalbuminuria among individuals with type 2 diabetes to be associated with a 2.4-fold increased risk for cardiovascular death as compared with normoalbuminuria. In addition, similar associations exist in hypertensive individuals (without diabetes) and in the general population (Jager et al. 1999). In the organism, nitrate and nitrite may function as an alternative source for nitric oxide (NO), an important and multifaceted physiological signaling molecule. Potential protective mechanisms related to cardiovascular diseases include vasodilation, inhibition of endothelial dysfunction, and inhibition of platelet aggregation. Endothelium-derived NO is an important signaling agent in the regulation of blood pressure (Tang et al. 2011). NO-mediated regulation of vascular tone involves increased cyclic guanosine monophosphate (cGMP) and subsequent relaxation of vascular smooth muscle (Tang et al. 2011). Nitric oxide suppresses systemic PAI-1 levels; elevated plasma PAI-1 levels are associated with endothelial dysfunction (Bouchie et al. 1998). Circulating PAI-1 levels are elevated in patients with type 2 diabetes, substantially contributing to the prothrombotic and proatherosclerotic changes in diabetes. In addition, plasma PAI-1 levels are elevated throughout the spectrum of insulin resistance, from the metabolic syndrome to prediabetes (period of impaired glucose tolerance) to diabetes (Pannacciulli et al. 2002).

Cellular Adhesion Molecules

Cellular adhesion molecules mediate the margination, adhesion, and transendothelial migration of circulating mononuclear cells from the blood stream to the extravascular compartment and have an important role in the progression of atherosclerotic plaque (Ross 1999). In addition, adhesion molecules and various other cytokines recruit and activate mononuclear cells to release matrix metalloproteinases, promoting plaque rupture and the initiation of acute coronary syndromes (Ross 1999).

Various inflammatory markers have been proposed to assist in the prediction of subsequent coronary events among healthy people (Ridker et al. 2000). Soluble intercellular adhesion molecule-1 and other adhesion molecules have been found to be associated with subsequent incidence of coronary heart disease among healthy men and women (Ridker et al. 2001), even if sICAM-1 was a better predictor for the presence of atherosclerosis and coronary heart disease than other adhesion molecules (Porsch-Oezçueruemez et al. 1999). Two prospective cohort studies, in fact, indicate that baseline levels of sICAM-1 are increased many years before a first myocardial infarction occurs (Ridker et al. 2000); data for sVCAM-1 are not so certain (de Lemos et al. 2000). Regarding sE-selectin, instead, it confirmed to be a reliable marker and to be strongly associated with traditional cardiovascular risk factors. E-selectin mediate leukocyte rolling on the endothelium and platelet-leukocyte interaction; it is only expressed in activated endothelial cells and acts as an adhesive reactant. On activation, sE-selectin is released into the circulation. Increased levels of sE-selectin have been found in individuals with myocardial infarction, and sE-selectin levels are related to blood pressure (Thorand et al. 2006).

Matrix Metalloproteinases

Matrix metalloproteinases are a family of proteolytic enzymes that are regulated by inflammatory signals to mediate changes in extracellular matrix. Humans have 24 matrix metalloproteinase genes including duplicated MMP-23 genes; thus there are 23 MMPs in humans. The activities of most MMPs are very low or negligible in the normal steady-state tissues, but expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell-cell and cell-matrix interaction. Matrix metalloproteinases are important in vascular remodeling, not only in the overall vasculature architecture but also, more importantly, in the advancing atherosclerotic plaque. Matrix metalloproteinases activation modifies the architecture of the plaque and may directly participate in the process of plaque rupture (Liu et al. 2006). Metalloproteinases are extremely potent protein degradation and modifying enzymes; thus, their biological actions are very tightly controlled by tissue inhibitor of metalloproteinases. Among MMPs, more specifically, MMP-2 and MMP-9 play an important role in vascular remodeling (Gibbons and Dzau 1994). MMP-2 and -9 are Zn^{+2} dependent endopeptidases, synthesized and secreted in zymogen form. The nascent form of the protein shows an N-terminal signal sequence ("pre" domain) that directs the protein to the endoplasmic reticulum. The pre domain is followed by a propeptide-"pro" domain that maintains enzyme latency until cleaved or disrupted and a catalytic domain that contains the conserved zinc-binding region. A hemopexin/vitronectin-like domain is also seen, that is connected to the catalytic domain by a hinge or linker region (Figs. 4, and 5). Increased MMP-2 and MMP-9 activity is associated with destruction of the elastic laminae of arteries and aneurysm formation in animals and humans (Longo et al. 2002). Metalloproteinase-2 and −9 resulted increased in patients with obesity (Derosa et al. 2008), hypertension (Derosa et al. 2006), type 2 diabetes (Derosa et al. 2007a), acute coronary syndrome (Derosa et al. 2007b, c, d). More recently, plasma MMP-9 levels have